Bivalent Probes of the Human Multidrug Transporter P-Glycoprotein

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Received April 25, 2006; Revised Manuscript Received June 28, 2006

ABSTRACT: A small library of bivalent agents was designed to probe the substrate binding sites of the human multidrug transporter P-glycoprotein (P-gp). The bivalent agents were composed of two copies of the P-gp substrate emetine, linked by tethers of varied composition. An optimum distance between the emetine molecules of approximately 10 Å was found to be necessary for blocking transport of the known fluorescent substrate rhodamine 123. Additionally, it was determined that hydrophobic tethers were optimal for bridging the bivalent compounds; hydrophilic or cationic moieties within the tether had a detrimental effect on inhibition of transport. In addition to acting as probes of P-gp's drug binding sites, these agents were also potent inhibitors of P-gp. One agent, EmeC5, had IC_{50} values of 2.9 μM for inhibiting transport of rhodamine 123 and approximately 5 nM for inhibiting the binding of a known P-gp substrate, [^{125}I]-iodoarylazidoprazosin. Although EmeC5 is an inhibitor of P-gp and was shown to interact directly with P-gp in one or more of the substrate binding sites, our data suggest that it is either not a P-gp transport substrate itself or a poor one. Most significantly, EmeC5 was shown to reverse the MDR phenotype of MCF-7/DX1 cells when co-administered with a cytotoxic agent, such as doxorubicin.

The successful treatment of cancer and AIDS is to a large degree dependent upon the effectiveness of therapeutic drugs. Unfortunately, most cancers either are intrinsically resistant to any initial treatment with such therapeutics or acquire resistance to a broad spectrum of these agents over time. Additionally, anti-AIDS therapeutics that target the protein HIV-1 protease have been found to be substrates for P-gp,[1] thereby leading to drug resistance with this class of molecules as well (*1*, *2*). It is well-established that this broad-based drug resistance results in large part from the over-expression of a plasma membrane polypeptide known as the multidrug transporter or P-glycoprotein (P-gp) (*3*−*6*). In addition, endogenous expression of P-gp in the gut and the blood−brain barrier can lead to the limited bioavailability of many drugs, further impeding successful chemotherapy (*7*−*10*).

Human P-gp is a member of a large superfamily of ATP-dependent proteins known as ABC (ATP-binding cassette) transporters. P-gp uses the energy of ATP hydrolysis to pump drug molecules out of cells so that they cannot elicit their cytotoxic effects (*4*−*6*, *11*−*13*). The P-gp transporter is an integral membrane protein comprised of two homologous halves each containing a cytosolic ATP binding site. ATP binding and hydrolysis are essential for the proper functioning of P-glycoprotein, including drug transport (*14*−*16*). The drug binding sites in P-gp, on the other hand, are localized to the two transmembrane domains. In one proposed model, P-gp reduces intracellular drug concentrations by acting as a "hydrophobic vacuum cleaner", effectively increasing drug efflux and decreasing drug influx by the recognition and removal of compounds from the membrane before they reach the cytosol to elicit their cytotoxic effects (Figure 1) (*4*, *17*).

P-gp confers resistance to a wide range of structurally and functionally unrelated compounds. The list of drugs that are substrates of P-gp includes a large majority of current pharmaceuticals, including the anticancer compounds daunomycin, doxorubicin, paclitaxel (*18*), and imatinib (Gleevec) (*19*), the immunosuppressant cyclosporin A (*20*−*22*), and agents that target HIV protease (*1*, *2*). Data from numerous groups support the hypothesis that there are at least two, if not more, substrate binding sites within the transporter domain of P-gp (*23*−*27*).

The pivotal role of P-gp in limiting drug accumulation in diseased cells suggests that inhibition of this drug transporter may be an attractive mode for reversing drug resistance and increasing the bioavailability of chemotherapeutic agents. To date, many inhibitors exist for P-gp, but most of these are also substrates and are transported. A small class of inhibitors has been reported, and they are believed to be weak substrates, or not substrates at all, including GF120918 (*28*), LY335979 (*29*), XR9576 (*30*), SCH66336 (*31*), and OC144-093 (*32*). Herein, we describe efforts to produce agents for probing and inhibiting P-gp by taking advantage of the multiple binding sites within the transporter domain with bivalent agents. There are numerous examples in nature and designed systems where polyvalency is used to promote tight binding that would otherwise not exist in a monovalent system (*33*, *36*). We have developed a novel bivalent agent based on monomeric emetine that is an inhibitor of P-gp transport in the low micromolar range.

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[1] Abbreviations: P-gp, P-glycoprotein; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DMSO, dimethyl sulfoxide; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; THF, tetrahydrofuran; RPMI, Roswell Park Memorial Institute; PBS, phosphate-buffered saline; IAAP, iodoarylazidoprazosin; R123, rhodamine 123; CsA, cyclosporin A; MDR, multidrug resistance.

10.1021/bi0608109 CCC: $33.50
Published on Web 08/31/2006

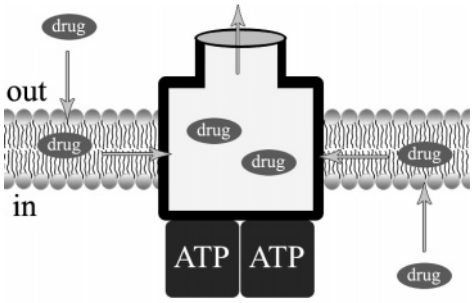


FIGURE 1: Model demonstrating how P-gp in the cell membrane can limit the accumulation of therapeutic agents within cells.

MATERIALS AND METHODS

Materials. Emetine hydrochloride and cyclosporin A were purchased from Fluka. Rhodamine 123 was purchased from Molecular Probes, Inc. (Eugene, OR). Doxorubicin hydrochloride was obtained from Oakwood Products (West Columbia, SC). [^{3}H]Daunamycin and [^{125}I]iodoarylazidoprazosin were purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without purification.

Typical Synthesis of Bivalent Emetine Compounds (EmeC4−C10 and EmeBE). To a solution of the commercially available diacid (0.07 mmol) in dry DMSO (5 mL) at room temperature were added HATU (0.28 mmol) and DIEA (0.28 mmol). After 10 min, the reaction flask was covered with aluminum foil, and emetine dihydrochloride (0.21 mmol) was added. The mixture was allowed to stir for 2 h, and the solvent was removed in vacuo. The residue was dissolved in ethyl acetate and washed with a saturated $NaHCO_3$ solution. The organic solvent was removed in vacuo, and the residue was dissolved in DMSO and filtered. The desired material was purified to homogeneity by reverse phase HPLC using a Vydac C8 column with an eluent consisting of solvent A (H_2O and 0.1% TFA) and solvent B (CH_3CN and 0.1% TFA) with a 60 min gradient consisting of 10 to 70% A, a flow rate of 8 mL/min, and monitoring at 214 and 280 nm. Each compound was characterized by ^{1}H and ^{13}C NMR and MALDI-TOF mass spectrometry (see the Supporting Information).

*Synthesis of Alloc-Protected Bis-Amine Diacid (**2**).* To a solution of bis-amine diacid **1** (1.76 g, 10 mmol) in a 1:1 THF/H_2O mixture (20 mL) at 0 °C were added allylchloroformate (3.62 g, 30 mmol) and NaOH (1.60 g, 40 mmol). The mixture was allowed to come to room temperature and stirred for 1 h. Additional NaOH (0.80 g, 20 mmol) was added, and the mixture was stirred at room temperature for an additional 1 h. The reaction mixture was washed with diethyl ether (twice) and acidified to pH ~3 with concentrated HCl. The mixture was extracted with ethyl acetate (three times), and the combined organic layers were dried over $MgSO_4$. The drying agent was filtered off, and the solvent was removed in vacuo. The compound was characterized by ^{1}H and ^{13}C NMR and MALDI-TOF mass spectrometry (see the Supporting Information).

*Synthesis of Alloc-Protected EmeBA (**3**).* To a solution of alloc-protected bis-amine diacid **2** (0.0240 g, 0.07 mmol) in dry DMSO (5 mL) at room temperature were added HATU (0.1064 g, 0.28 mmol) and DIEA (0.0362 g, 0.28 mmol). After 10 min, the reaction flask was covered with aluminum foil and emetine dihydrochloride (0.1018 g, 0.21 mmol) was added. The mixture was allowed to stir for 2 h, and the solvent was removed in vacuo. The residue was dissolved in ethyl acetate and washed with a saturated $NaHCO_3$ solution. The organic solvent was removed in vacuo, and the residue was dissolved in DMSO and filtered. The desired material was purified to homogeneity by reverse phase HPLC using a Vydac C8 column as described above. HPLC t_R = 38.0 min. The compound was characterized by ^{1}H and ^{13}C NMR and MALDI-TOF mass spectrometry.

Alloc-protected EmeBA **3** (30.0 mg, 0.024 mmol) was dissolved in a solution of dry MeOH and DCM (1:2, 5 mL), and the reaction flask was covered with aluminum foil. A catalytic amount of $Pd(Ph_3)_4$ (13.86 mg, 0.012 mmol) was added to the mixture with morpholine (0.1220 g, 1.4 mmol). The reaction mixture was stirred for 2 h, and the solvent was removed in vacuo. The residue was dissolved in DMSO and filtered. The desired material was purified to homogeneity by reverse phase HPLC using a Vydac C8 column as described above. The compound was characterized by ^{1}H and ^{13}C NMR and MALDI-TOF mass spectrometry.

Cell Culture. All cell lines were cultured at 37 °C with 5% CO_2. MCF-7 cells (breast adenocarcinoma) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Cambrex Bio Science Walkersville, Inc.), 2 mM L-glutamine (Cellgro, Mediatech), and 50 units/mL penicillin and 50 μg/mL streptomycin (Cellgro, Mediatech). The MCF-7/DX1 cell line overexpressing P-gp was maintained in the same medium as the parental MCF-7 with the addition of 1 μM doxorubicin to the culture medium.

Flow Cytometry Assay. Substrate accumulation assays were performed as described previously with minor modifications (*34*). For substrate accumulation studies using cell suspensions, 500 000 cells were incubated with either 0.5 μg/mL rhodamine 123 alone or in the presence of varying concentrations of standard inhibitors or synthesized compounds for 45 min at 37 °C. The cells were harvested by centrifugation at 300*g* and resuspended in fresh medium with or without inhibitor and incubated for an additional 45 min at 37 °C. The cells were harvested by centrifugation at 300*g*; the medium was removed, and the cells were resuspended in 400 μL of PBS. The cells were kept on ice until analysis. For assays performed in a 24-well plate, each well was evenly seeded and the cells were allowed to grow until they were 70−80% confluent and assayed under similar conditions except that the cells remained adhered to the plate. After the incubations, the cells were trypsinized gently, resuspended in culture medium, and kept on ice until analysis. All cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 nm argon laser and a 530 nm band-pass filter (FL1) for rhodamine 123. Ten thousand (10 000) cells were counted for each data point. The fluorescence data are expressed as the mean of arbitrary fluorescence units derived from histogram plots of the 10 000 cells that were examined. All assays were performed in triplicate, and IC_{50} values were obtained by fitting the concentration-dependent data using SigmaPlot.

Radioactive Substrate Assay. Radioactive substrate accumulation assays were performed as described previously except that 24-well plates were used and seeded with 100 000

cells on the day prior to the assay (*34*). All assays were performed in triplicate, and IC_{50} values were obtained by fitting the concentration-dependent data using SigmaPlot.

Membrane Preparation. MCF-7/DX1 cells were harvested by scraping 48 h after plating. Crude membrane extracts were prepared as described previously (*34*) except that the membrane fraction was harvested by centrifugation at 300000*g* for 40 min at 4 °C. The membrane pellet was collected, and the membranes were assayed for total protein concentration, aliquoted, frozen on dry ice, and kept at −80 °C until they were used.

ATPase Assay. Crude membranes derived from MCF-7/DX1 cells were analyzed for both vanadate-sensitive basal and drug-stimulated ATP consumption in the absence and presence of increasing concentrations of EmeC5. Activity was measured by the colorimetric detection of inorganic phosphate release at 880 nm, as described previously (*34*). Verapamil-stimulated activity was assayed in the presence of 30 μM verapamil with or without increasing concentrations of EmeC5. All assays were performed in triplicate.

IAAP Photoaffinity Labeling. [^{125}I]Iodoarylazidoprazosin ([^{125}I]IAAP) (specific activity of 2200 Ci/mmol) was used to label P-gp as described previously (*34*). The reaction scale was reduced to a total volume of 50 μL. The crude membranes (30 μg) were incubated at room temperature in 50 mM Tris-HCl (pH 7.5) with IAAP (18 nM) for 5 min under subdued light. The samples were then illuminated with a UV lamp assembly fitted with two black light UV-A long wave tubes (365 nm) for 20 min on ice. Membrane protein (10 μg) was subjected to SDS−PAGE on a 7.5% Tris glycine gel, fixed, dried overnight, and exposed to Bio-Max MR film (Eastman Kodak Co.) at −80 °C for 12−24 h. To determine the amount of [^{125}I]IAAP photo-cross-linked to P-gp, each band was quantified from the dried gels using SigmaScan. Values were expressed either in arbitrary units or as a percentage of a control experiment.

Persistence of Inhibition of P-gp-Mediated Efflux. The day prior to the assay, 100 000 cells were seeded in a 12-well culture plate. The following day, the cells were washed three times with PBS and incubated with 0.5 mg/mL rhodamine 123 with the inhibitor at the desired concentration at 37 °C. After 40 min, cells were washed three times with room-temperature PBS and incubated with culture medium not containing either R123 or the inhibitor. The efflux phase was carried out at 37 °C. The medium was removed, and the cells were removed by mild trypsinization and diluted with 400 mL of cold PBS. Cells were analyzed at the indicated time points using flow cytometry. A minimum of 10 000 events were collected per data point, and each data point was determined in triplicate.

RESULTS AND DISCUSSION

Numerous studies have pointed to the existence of at least two spatially distinct substrate binding sites within the transmembrane domain of P-gp that function in transport or regulation of transport (*23*−*27*, *35*). This multiplicity of binding pockets provides the opportunity to both probe and inhibit P-gp transport with multivalent agents. Along these lines, Sauna et al. (*36*) elegantly designed stipiamide homodimers capable of inhibiting P-gp. They evaluated dimers that were cross-linked with hydrophilic PEG-based tethers and found

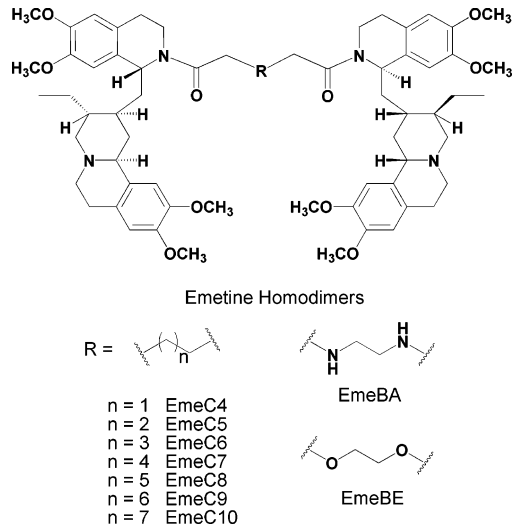


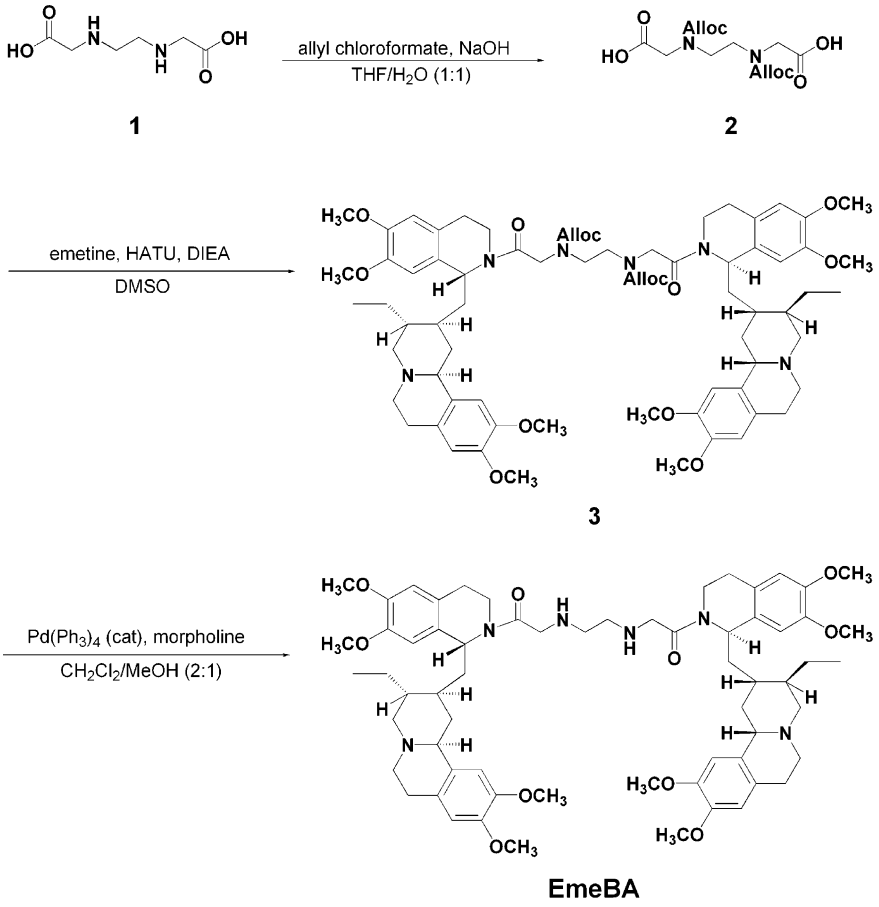
FIGURE 2: Structures of the designed bivalent emetine compounds with varying cross-linking moieties.

an optimum tether length of approximately 35 Å. To more fully probe the P-gp transporter, we sought a more universal substrate of P-gp, with features in common with larger classes of substrates that could be easily dimerized with tethers of varied composition.

Initially, our search for an appropriate P-gp substrate focused on compounds that contained the bicyclic recognition element found in many of the strongest P-gp inhibitors (*3*, *37*−*39*). The compound emetine appeared to complement our needs the most. Emetine is a known substrate of P-gp (*40*), and it contains a secondary amine for cross-linking but also contains a tertiary amine, an important recognition element of P-gp, that would remain unmodified (*39*). Emetine is used in the treatment of human amoebiasis and is one of the few antiparasitic compounds available (*41*). Emetine suffers from poor intracellular accumulation in resistant parasites because it is a substrate for a parasitic form of P-gp (*42*). Therefore, novel emetine-based inhibitors may be useful for the treatment of amoebiasis, in addition to proving useful for studying the pharmacophore of P-gp.

One of the more important design features for bivalent agents is the tether linking the ligands. In an attempt to produce an unbiased small library, the character of the tether was diversified (Figure 2). Among the characteristics that were varied were length, degree of hydrophobicity, and charge. The library of emetine homodimers was designed to contain nine different compounds that should provide data about the distances between binding sites in P-gp and the nature of the functionality between these sites. One series of compounds contains methylene-based tethers of varying lengths. Compounds EmeC4−C10 have maximum distances between the cross-linked, nitrogen atoms on emetine of approximately 9−16 Å. These distances were chosen on the basis of the finding of Loo and Clarke that the binding pocket of the P-gp is 9−25 Å long (*43*). We chose to keep the tethers flexible in this initial study, but the results that were obtained should assist in the design of more rigid moieties in further defining the spatial arrangement of the binding sites. Little is known about the environment between the different binding sites of P-gp, but recently, the drug binding pocket of P-gp has been shown to be accessible to water (*44*). These

Scheme 1: Synthesis of EmeBA



data imply that perhaps a more hydrophilic tether would be better accommodated in the emetine homodimers. Therefore, we chose to synthesize compounds containing amino groups and ether moieties in the tether as well (Figure 2).

The bivalent emetine derivatives were prepared starting with emetine and the required bis-carboxylic acids, with HATU and DIEA. Commercially available diacids were used for compounds EmeC4−C10, EmeBA, and EmeBE. Prior to the synthesis of EmeBA, the bis-amino diacid (Scheme 1) was alloc-protected, thereby necessitating a deprotection step after the coupling reaction. All final compounds were purified to homogeneity by reverse phase HPLC and analyzed by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry.

To assess the function of P-gp and the effectiveness of our designed agents as inhibitors, we employed two human carcinoma lines. An MCF-7 human breast cancer parental cell line was used as a negative control because it does not express detectable levels of P-gp. A second multidrug-resistant (MDR) subline derived from MCF-7 cells, MCF-7/DX1, was used because it is 200-fold more resistant to doxorubicin than the MCF-7 cell line and has been shown to overexpress P-gp by immunoblot analysis (data not shown). Furthermore, the MCF-7/DX1 cells did not express detectable amounts of ABCG2, as determined by immunoblot analysis (data not shown). To assess function, a fluorescent substrate of P-gp, rhodamine 123 (R123), was incubated with both cell lines and flow cytometry was used to assess the accumulation of fluorescence within the cells (Figure 3). The MCF-7/DX1 cells that express P-gp do not accumulate R123 (Figure 3, DMSO), demonstrating that P-gp is effluxing the compound out of the cells.

To determine the efficacy of our designed agents, R123 accumulation was assayed in the presence and absence of each of the library members (10 μM). The known P-gp modulator cyclosporin A (CsA) was used as a positive control. An increased level of cellular fluorescence associated with the MCF-7/DX1 cell line is indicative of inhibition of P-gp transport. The parental MCF-7 cell line was also assayed with and without the designed compounds, and the

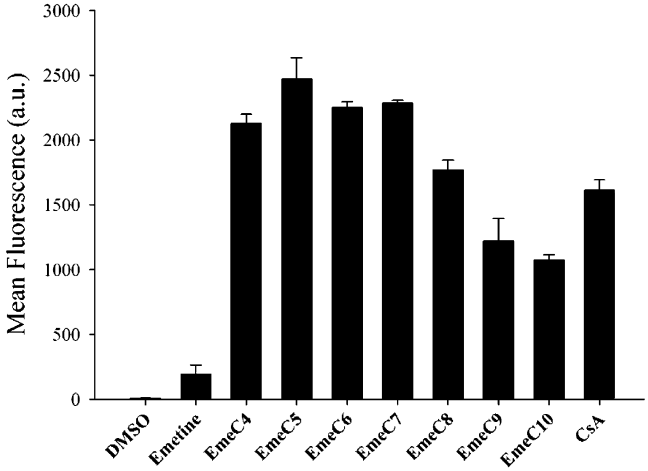


FIGURE 3: Flow cytometry data for MCF-7/DX1 cells treated with R123 (0.5 μg/mL), DMSO, emetine, each of the bivalent emetine agents with hydrophobic tethers, and cyclosporin A (CsA) (10 μM each).

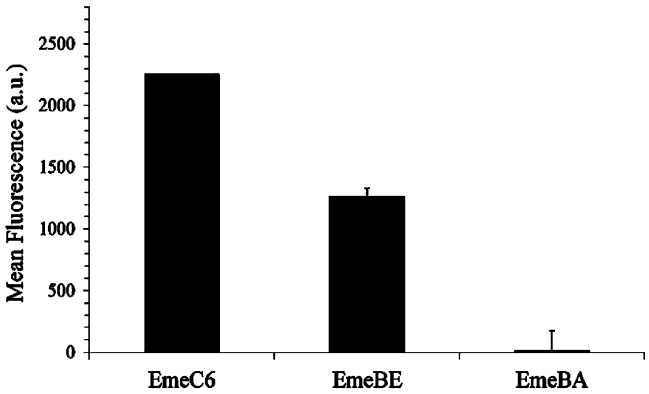


FIGURE 4: Flow cytometry data for MCF-7/DX1 cells treated with R123 (0.5 μg/mL) and bivalent emetine agents with hydrophobic (EmeC6), hydrophilic (EmeBE), and charged (EmeBA) tethers (10 μM each).

data confirmed that accumulation of R123 was significant and remained unaffected by the addition of the library members (data not shown). For the MCF-7/DX1 cells, we found that the addition of library members with a hydrophobic tether resulted in accumulation of R123 and thus increased cellular fluorescence (Figure 3). When the emetine homodimer EmeC5, for example, was co-administered with R123, a higher level of cellular fluorescence was observed as with the positive control, cyclosporin A (Figure 3). Similar results were also obtained with the fluorescent substrates calcein-AM and bodipy-prazosin (data not shown). These data demonstrate that our inhibitors are blocking R123 efflux from the MCF-7/DX1 P-gp-expressing cells. These data also indicate that the bivalent compounds are more potent than the emetine monomer. For example, EmeC5 is 12-fold more potent than emetine at a concentration of 10 μM, thereby supporting the hypothesis that both halves of the homodimeric agents are interacting with P-gp binding sites. Likewise, EmeC5 was found to be 26-fold more potent than emetine at a concentration of 10 μM with a KB cell line that expresses P-gp (data not shown).

The extent of P-gp inhibition was also found to be dependent on the length of the tether and its hydrophobic nature. Relatively equivalent cellular fluorescence was observed with 10 μM of compounds EmeC4−C7, with the best potency found with EmeC5 (Figure 3). Further lengthening the tether chain beyond C7, however, led to a decreased level of retention of R123 in the MCF-7/DX1 cells, indicating weaker inhibition of P-gp. Although a length dependence is observed, it should be noted that even compounds with nonoptimal tether lengths still had modest inhibition activity. These data provide further evidence that the two emetine moieties are being recognized by P-gp. Also, addition of hydrophilic ether or charged amino groups to the tether led to diminished (EmeBE) and minimal (EmeBA) cellular fluorescence as compared to that of EmeC6 (Figure 4). These data may be due to a disfavored interaction between EmeBE and EmeBA with the transporter or a disinclination of the compounds to partition into the membrane as compared to the carbon-based tethers. There is little difference in the molecular weights of the dimers that have been studied (Table 1), but logP values are severely reduced for EmeBA. Therefore, it is possible that the reduced lipophilicity of EmeBA is precluding membrane partitioning. Overall, our data indicate that the optimal spacing between the two emetine moieties for interaction with the P-gp transporter is approximately 10 Å, and the functionality in the region spanning the two binding sites can accommodate hydrophobic moieties. These results are interesting when compared to the data obtained by Sauna et al., as they found that much longer (35 Å) and hydrophilic (PEG) tethers linking stipiamide monomers provided optimal inhibition (*36*). It is possible that the stipiamide homodimers occupy binding sites orthogonal from that of the emetine homodimers and that the location of these sites and the functionality bridging these sites is significantly altered from the emetine binding pockets.

To further support the fluorescence data, experiments with a radiolabeled substrate of P-gp, [^{3}H]daunomycin, were also performed with the MCF-7/DX1 cell line. The extent of [^{3}H]-daunomycin retention in cells as a function of an increase in EmeC5 concentration was investigated (Figure 5). An increasing level of accumulation of [^{3}H]daunomycin was observed with EmeC5 in a concentration-dependent manner, confirming the inhibition of P-gp in these cells. There is excellent agreement in the concentration-dependent data with EmeC5 using both fluorescent and radioactive substrates (Figure 5). Therefore, IC_{50} values were obtained for emetine and EmeC4−C6 using the fluorescence assay with R123

Table 1: Chemical Properties of Emetine and Emetine Dimers

	molecular weight	tether length (Å)[a]	charge	logP[b]
emetine	481	−	+2	5.48
EmeC4	1071	8.7	+2	8.37
EmeC5	1085	10.0	+2	8.40
EmeC6	1099	11.3	+2	8.42
EmeC7	1113	12.5	+2	8.43
EmeC8	1127	13.8	+2	8.46
EmeC9	1142	15.1	+2	8.48
EmeC10	1156	16.3	+2	8.49
EmeBE	1103	10.8	+2	7.64
EmeBA	1101	10.9	+4	4.56

[a] Distance between the amide nitrogens. [b] Calculated logP.

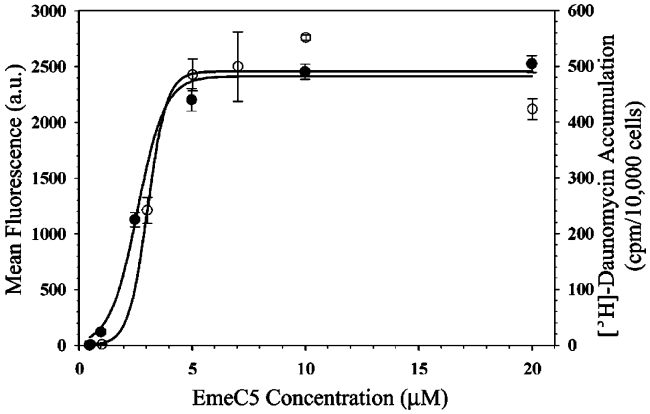


FIGURE 5: Dependence of EmeC5 concentration on accumulation of the fluorescent substrate R123 (*y*-axis, left, ○) and the radiolabeled substrate [^{3}H]daunomycin (*y*-axis, right, ●) with MCF-7/DX1 cells.

Table 2: Comparison of Efflux Inhibition of R123

	IC_{50}[a] (μM)		IC_{50}[a] (μM)
emetine	33 ± 1.4	EmeC6	3.5 ± 0.7
EmeC4	2.6 ± 0.2	CsA	5.3 ± 1.4
EmeC5	2.9 ± 0.2		

[a] The maximal cellular fluorescence for each compound was used to calculate the IC_{50} value.

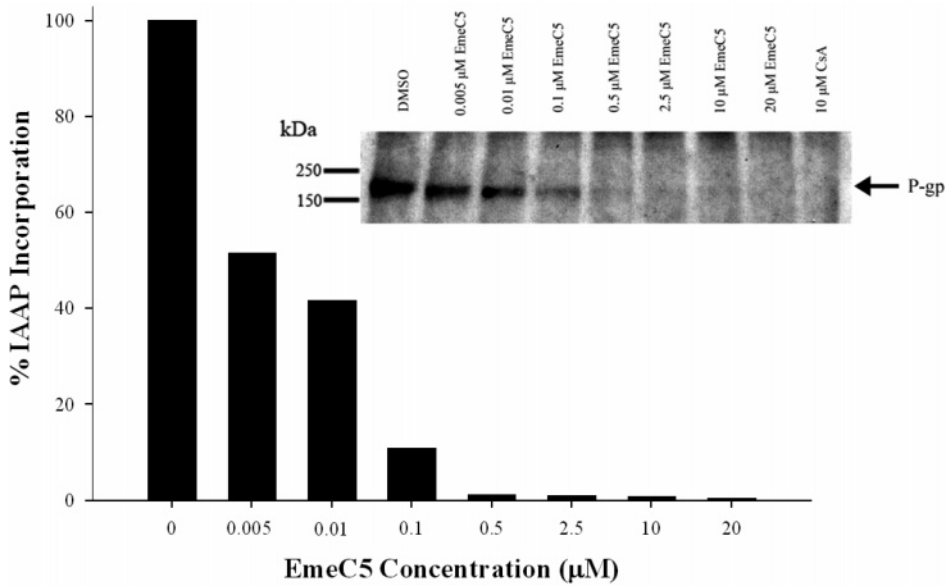


FIGURE 6: Extent of photoaffinity cross-linking of IAAP with P-gp as a function of increasing EmeC5 concentration. The inset shows the autoradiography of the SDS−PAGE analysis of the reaction mixtures (cyclosporin A was added as a positive control).

(Table 2). The homodimers were found to be ~10-fold more potent than emetine itself.

As further evidence that the homodimeric emetine derivatives are binding to the transporter region of P-gp, photoaffinity cross-linking experiments were performed. In these experiments, we chose to use one of the best agents from the R123 screen, EmeC5, to probe mechanistic issues of inhibition. A photoactive azido analogue of the known P-gp substrate prazosin, containing an ^{125}I label ([^{125}I]IAAP), was incubated with crude membrane extracts of P-gp in the presence and absence of increasing concentrations of EmeC5, and the mixture was exposed to UV light (365 nm). SDS−PAGE analysis of the resulting mixture (Figure 6) demonstrated that EmeC5 competed for the [^{125}I]IAAP binding site in a concentration-dependent manner and suggests that EmeC5 does, in fact, interact with P-gp in the substrate binding site(s). Upon quantification using SigmaScan, we found that EmeC5 inhibits prazosin cross-linking with an IC_{50} value of approximately 5 nM. Although it is tempting to attempt to correlate the IC_{50} values of EmeC5 for transport and binding, it is important to keep in mind that the concentrations of substrate in each of these experiments are significantly different (18 nM and 1.3 μM, respectively), and thus, direct comparisons are not appropriate.

As previously discussed, most P-gp inhibitors are also substrates of P-gp and are transported out of the cell. To determine if EmeC5 is also a substrate of P-gp, two assays were employed. The ability of a drug to stimulate the ATPase activity of P-gp is generally regarded as a good measure of whether the compound is transported (*45*, *46*). Therefore, using crude membrane preparations derived from MCF-7/DX1 cells, we assessed the ability of EmeC5 to stimulate the basal ATPase activity of P-gp in a concentration-dependent manner. We also assessed the ability of EmeC5 to inhibit ATPase activity when the transporter is already stimulated by a known substrate, in this case, verapamil (Figure 7). We demonstrated that in the presence of increasing concentrations of EmeC5 alone, the basal ATPase activity was not stimulated and, in fact, was inhibited by EmeC5. This trend suggests that EmeC5 either is not a substrate or is a weak substrate of P-gp and actually inhibits the basal level of P-gp ATPase activity that is thought to result from endogenous substrates. EmeC5 was also able to inhibit verapamil (30 μM)-stimulated ATPase activity in a concentration-dependent manner. At 1 μM EmeC5, the level of ATP hydrolysis with verapamil was returned to basal levels.

To further probe if EmeC5 is a substrate of P-gp, R123 retention experiments were performed using GF120918 and CsA as positive and negative controls. GF120918 is an known inhibitor of P-gp that is not considered to be a substrate (*28*), whereas CsA is known to be both an inhibitor and a substrate (*47*). Each of these molecules was co-incubated with R123 in MCF-7/DX1 cells; the compounds were removed from the media, and the amount of R123 retained in the cells was monitored by flow cytometry as a function of time. As previously described (*28*), R123 is retained well in GF120918-treated cells, indicating that GF120918 is not transported out of the cells during the course of the experiment. The opposite trend is observed with CsA. In CsA-treated cells, R123 is poorly retained, presumably due to transport of CsA out of the cells along with R123 by P-gp. Interestingly, EmeC5 is intermediate in its ability to allow cells to retain R123 as compared to GF120918 and CsA, suggesting that EmeC5 may be a weak substrate of P-gp. Similar data have been obtained for other P-gp inhibitors, such as OC144-093 (*32*), LX335979 (*29*), and XR9576 (*30*). With these inhibitors, retention of the substrate upon removal of inhibitor was also observed, suggesting that the inhibitor itself is not being efficiently transported.

As a final set of experiments, we were interested if it would be possible to reverse the drug-resistant phenotype of the MCF-7/DX1 cell line with EmeC5 and return doxorubicin cytotoxicity to that observed in the parental MCF-7 drug-sensitive cells. Co-administration of noncytotoxic levels of EmeC5 (0−5 μM) and increasing amounts of doxorubicin to both the MCF-7 parental cell line and the MCF-7/DX1 subline was performed, and IC_{50} values for doxorubicin cytotoxicity were determined using a MTT assay (Table 3). EmeC5 had no effect on the IC_{50} value of doxorubicin in MCF-7 parental cells but dramatically

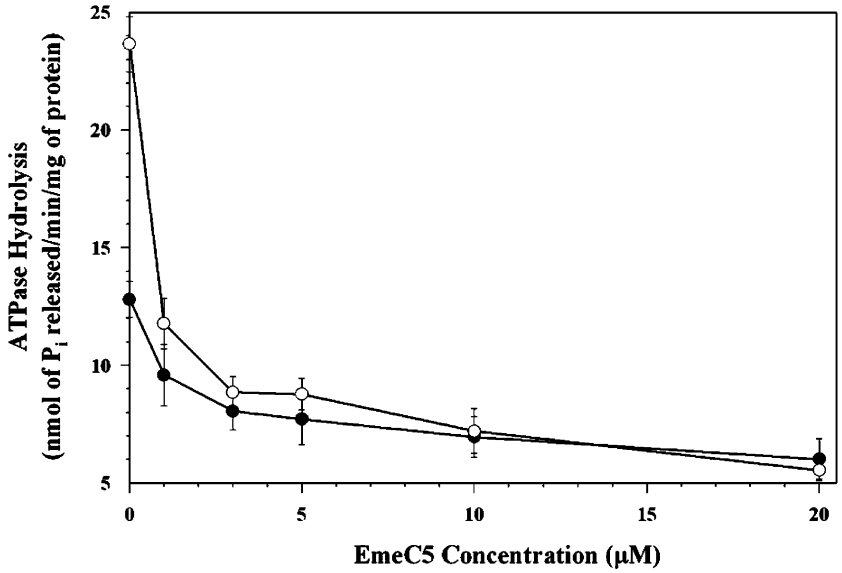


FIGURE 7: Extent of ATP hydrolysis by crude membrane extracts derived from MCF-7/DX1 cells overexpressing P-gp. Increasing concentrations of EmeC5 alone (●) and verapamil (30 μM) with increasing concentrations of EmeC5 (○).

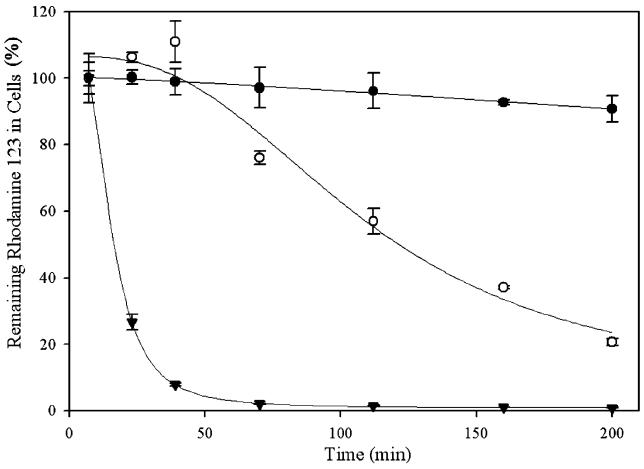


FIGURE 8: Retention of R123 in MCF-7/DX1 cells in the presence of known inhibitors and EmeC5. After a 40 min incubation of MCF-7/DX1 cells with R123 and EmeC5 at 10 μM (○), CsA at 10 μM (▼), or GF120918 at 3 μM (●), the cells were washed and incubated in fresh medium without R123 or inhibitors for the indicated times.

Table 3: IC_{50} Values for Doxorubicin with Co-Administration of EmeC5

	0 μM EmeC5	2.5 μM EmeC5	5 μM EmeC5
MCF-7	0.18 ± 0.01 μM	0.24 ± 0.01 μM	0.27 ± 0.02 μM
MCF-7/DX1	49.7 ± 1.7 μM	8.2 ± 0.3 μM	1.2 ± 0.1 μM

enhanced the effect of doxorubicin on the DX1 subline in a dose-dependent manner. A 50-fold increase in the cytotoxicity of doxorubicin was observed with 5 μM EmeC5, a concentration that was found to be noncytotoxic (data not shown). These data suggest that bivalent inhibitors of P-gp transport can effectively block drug resistance pathways involving P-gp and can restore the effectiveness of cytotoxic agents to P-gp-overexpressing cells.

We have demonstrated that bivalent agents containing the P-gp substrate emetine and a variety of cross-linking agents can provide useful information concerning the separation of substrate binding sites within the P-gp transporter and the nature of the environment between these sites. In addition to acting as biochemical probes of P-gp, these agents were also potent inhibitors of P-gp. One agent, EmeC5, had IC_{50} values of 2.9 μM for inhibiting transport and approximately 5 nM for inhibiting the binding of a known P-gp substrate. Although EmeC5 is an inhibitor of P-gp and was shown to occupy a known substrate binding site, our data suggest that it is only a weak substrate of P-gp itself. Most importantly, EmeC5 was shown to reverse the MDR phenotype of MCF-7/DX1 cells when co-administered with a cytotoxic agent such as doxorubicin. These experiments bode well for future design efforts with multivalent agents in further probing the binding sites of P-gp and enhancing the efficacy of inhibitors.

ACKNOWLEDGMENT

We thank Karin Ejendal for helpful advice and training. This paper is dedicated to Professor Ronald Breslow on the occasion of his 75th birthday.

SUPPORTING INFORMATION AVAILABLE

All spectral characterization for the synthetic agents. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Kim, R. B., Fromm, M. F., Wandel, C., Leake, B., Wood, A. J., Roden, D. M., and Wilkinson, G. R. (1998) The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors, *J. Clin. Invest. 101*, 289−94.
2. Lee, C. G., Gottesman, M. M., Cardarelli, C. O., Ramachandra, M., Jeang, K. T., Ambudkar, S. V., Pastan, I., and Dey, S. (1998) HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter, *Biochemistry 37*, 3594−601.
3. Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter, *Annu. Rev. Pharmacol. Toxicol. 39*, 361−98.
4. Gottesman, M. M., and Pastan, I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter, *Annu. Rev. Biochem. 62*, 385−427.
5. Gottesman, M. M., Hrycyna, C. A., Schoenlein, P. V., Germann, U. A., and Pastan, I. (1995) Genetic analysis of the multidrug transporter, *Annu. Rev. Genet. 29*, 607−49.

6. Hrycyna, C. A. (2001) Molecular genetic analysis and biochemical characterization of mammalian P-glycoproteins involved in multidrug resistance, *Semin. Cell Dev. Biol. 12*, 247−56.
7. Gottesman, M. M., Fojo, T., and Bates, S. E. (2002) Multidrug resistance in cancer: Role of ATP-dependent transporters, *Nat. Rev. Cancer 2*, 48−58.
8. Huisman, M. T., Smit, J. W., Wiltshire, H. R., Hoetelmans, R. M., Beijnen, J. H., and Schinkel, A. H. (2001) P-Glycoprotein limits oral availability, brain, and fetal penetration of saquinavir even with high doses of ritonavir, *Mol. Pharmacol. 59*, 806−13.
9. Loscher, W., and Potschka, H. (2005) Drug resistance in brain diseases and the role of drug efflux transporters, *Nat. Rev. Neurosci. 6*, 591−602.
10. Schinkel, A. H., and Jonker, J. W. (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: An overview, *Adv. Drug Delivery Rev. 55*, 3−29.
11. Borst, P., and Elferink, R. O. (2002) Mammalian ABC transporters in health and disease, *Annu. Rev. Biochem. 71*, 537−92.
12. Gottesman, M. M., and Pastan, I. (1988) The multidrug transporter, a double-edged sword, *J. Biol. Chem. 263*, 12163−6.
13. Higgins, C. F. (1992) ABC transporters: From microorganisms to man, *Annu. Rev. Cell Biol. 8*, 67−113.
14. Hrycyna, C. A., Ramachandra, M., Germann, U. A., Cheng, P. W., Pastan, I., and Gottesman, M. M. (1999) Both ATP sites of human P-glycoprotein are essential but not symmetric, *Biochemistry 38*, 13887−99.
15. Urbatsch, I. L., Beaudet, L., Carrier, I., and Gros, P. (1998) Mutations in either nucleotide-binding site of P-glycoprotein (Mdr3) prevent vanadate trapping of nucleotide at both sites, *Biochemistry 37*, 4592−602.
16. Loo, T. W., and Clarke, D. M. (1995) Covalent modification of human P-glycoprotein mutants containing a single cysteine in either nucleotide-binding fold abolishes drug-stimulated ATPase activity, *J. Biol. Chem. 270*, 22957−61.
17. Raviv, Y., Pollard, H. B., Bruggemann, E. P., Pastan, I., and Gottesman, M. M. (1990) Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells, *J. Biol. Chem. 265*, 3975−80.
18. Sparreboom, A., van Asperen, J., Mayer, U., Schinkel, A. H., Smit, J. W., Meijer, D. K., Borst, P., Nooijen, W. J., Beijnen, J. H., and van Tellingen, O. (1997) Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine, *Proc. Natl. Acad. Sci. U.S.A. 94*, 2031−5.
19. Illmer, T., Schaich, M., Platzbecker, U., Freiberg-Richter, J., Oelschlagel, U., von Bonin, M., Pursche, S., Bergemann, T., Ehninger, G., and Schleyer, E. (2004) P-Glycoprotein-mediated drug efflux is a resistance mechanism of chronic myelogenous leukemia cells to treatment with imatinib mesylate, *Leukemia 18*, 401−8.
20. Archinal-Mattheis, A., Rzepka, R. W., Watanabe, T., Kokubu, N., Itoh, Y., Combates, N. J., Bair, K. W., and Cohen, D. (1995) Analysis of the interactions of SDZ PSC 833 ([3′-keto-Bmt1]-Val2]-Cyclosporine), a multidrug resistance modulator, with P-glycoprotein, *Oncol. Res. 7*, 603−10.
21. Farrell, R. J., Menconi, M. J., Keates, A. C., and Kelly, C. P. (2002) P-Glycoprotein-170 inhibition significantly reduces cortisol and ciclosporin efflux from human intestinal epithelial cells and T lymphocytes, *Aliment. Pharmacol. Ther. 16*, 1021−31.
22. Tsuji, A., Tamai, I., Sakata, A., Tenda, Y., and Terasaki, T. (1993) Restricted transport of cyclosporin A across the blood-brain barrier by a multidrug transporter, P-glycoprotein, *Biochem. Pharmacol. 46*, 1096−9.
23. Dey, S., Ramachandra, M., Pastan, I., Gottesman, M. M., and Ambudkar, S. V. (1997) Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein, *Proc. Natl. Acad. Sci. U.S.A. 94*, 10594−9.
24. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2003) Methanethiosulfonate derivatives of rhodamine and verapamil activate human P-glycoprotein at different sites, *J. Biol. Chem. 278*, 50136−41.
25. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2003) Simultaneous binding of two different drugs in the binding pocket of the human multidrug resistance P-glycoprotein, *J. Biol. Chem. 278*, 39706−10.
26. Shapiro, A. B., and Ling, V. (1997) Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities, *Eur. J. Biochem. 250*, 130−7.
27. Martin, C., Berridge, G., Higgins, C. F., Mistry, P., Charlton, P., and Callaghan, R. (2000) Communication between multiple drug binding sites on P-glycoprotein, *Mol. Pharmacol. 58*, 624−32.
28. Hyafil, F., Vergely, C., Du Vignaud, P., and Grand-Perret, T. (1993) In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative, *Cancer Res. 53*, 4595−602.
29. Dantzig, A. H., Shepard, R. L., Cao, J., Law, K. L., Ehlhardt, W. J., Baughman, T. M., Bumol, T. F., and Starling, J. J. (1996) Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979, *Cancer Res. 56*, 4171−9.
30. Mistry, P., Stewart, A. J., Dangerfield, W., Okiji, S., Liddle, C., Bootle, D., Plumb, J. A., Templeton, D., and Charlton, P. (2001) In vitro and in vivo reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576, *Cancer Res. 61*, 749−58.
31. Wang, E., Casciano, C. N., Clement, R. P., and Johnson, W. W. (2001) The farnesyl protein transferase inhibitor SCH66336 is a potent inhibitor of MDR1 product P-glycoprotein, *Cancer Res. 61*, 7525−9.
32. Newman, M. J., Rodarte, J. C., Benbatoul, K. D., Romano, S. J., Zhang, C., Krane, S., Moran, E. J., Uyeda, R. T., Dixon, R., Guns, E. S., and Mayer, L. D. (2000) Discovery and characterization of OC144-093, a novel inhibitor of P-glycoprotein-mediated multidrug resistance, *Cancer Res. 60*, 2964−72.
33. Whitesides, G. M., Mammen, M., and Choi, S. (1998) Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. *Angew. Chem., Int. Ed. 37*, 2754−94.
34. Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. (1998) Functional expression of human P-glycoprotein from plasmids using vaccinia virus-bacteriophage T7 RNA polymerase system, *Methods Enzymol. 292*, 456−73.
35. Wang, E. J., Casciano, C. N., Clement, R. P., and Johnson, W. W. (2000) Two transport binding sites of P-glycoprotein are unequal yet contingent: Initial rate kinetic analysis by ATP hydrolysis demonstrates intersite dependence, *Biochim. Biophys. Acta 1481*, 63−74.
36. Sauna, Z. E., Andrus, M. B., Turner, T. M., and Ambudkar, S. V. (2004) Biochemical basis of polyvalency as a strategy for enhancing the efficacy of P-glycoprotein (ABCB1) modulators: Stipiamide homodimers separated with defined-length spacers reverse drug efflux with greater efficacy, *Biochemistry 43*, 2262−71.
37. Ambudkar, S. V., Kimchi-Sarfaty, C., Sauna, Z. E., and Gottesman, M. M. (2003) P-Glycoprotein: From genomics to mechanism, *Oncogene 22*, 7468−85.
38. Robert, J., and Jarry, C. (2003) Multidrug resistance reversal agents, *J. Med. Chem. 46*, 4805−17.
39. Wang, R. B., Kuo, C. L., Lien, L. L., and Lien, E. J. (2003) Structure-activity relationship: Analyses of P-glycoprotein substrates and inhibitors, *J. Clin. Pharm. Ther. 28*, 203−28.
40. Polli, J. W., Wring, S. A., Humphreys, J. E., Huang, L., Morgan, J. B., Webster, L. O., and Serabjit-Singh, C. S. (2001) Rational Use of in Vitro P-Glycoprotein Assays in Drug Discovery, *J. Pharmacol. Exp. Ther. 299*, 620−8.
41. Wolfe, M. S. (1982) The treatment of intestinal protozoan infections, *Med. Clin. N. Am. 66*, 707−20.
42. Ghosh, S. K., Lohia, A., Kumar, A., and Samuelson, J. (1996) Overexpression of P-glycoprotein gene 1 by transfected *Entamoeba histolytica* confers emetine-resistance, *Mol. Biochem. Parasitol. 82*, 257−60.
43. Loo, T. W., and Clarke, D. M. (2001) Determining the dimensions of the drug-binding domain of human P-glycoprotein using thiol cross-linking compounds as molecular rulers, *J. Biol. Chem. 276*, 36877−80.
44. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2004) The drug-binding pocket of the human multidrug resistance P-glycoprotein is accessible to the aqueous medium, *Biochemistry 43*, 12081−9.
45. Borgnia, M. J., Eytan, G. D., and Assaraf, Y. G. (1996) Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its atpase activity, *J. Biol. Chem. 271*, 3163−71.
46. Al-Shawi, M. K., and Senior, A. E. (1993) Characterization of the adenosine triphosphatase activity of Chinese hamster P-glycoprotein, *J. Biol. Chem. 268*, 4197−206.
47. Farrell, R. J., Menconi, M. J., Keates, A. C., and Kelly, C. P. (2002) P-Glycoprotein-170 inhibition significantly reduces cortisol and cyclosporin efflux from human intestinal epithelial cells and T lymphocytes, *Aliment. Pharmacol. Ther. 16*, 1021−31.

BI0608109